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An Updated Evaluation of Citrus Fruit (*Citrus* spp.) as a Pathway for the Introduction of Citrus Canker Disease (*Xanthomonas axonopodis* pv. *citri*)

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Executive Summary

This document updates the characterization of risk described in USDA (2007a), "Evaluation of asymptomatic citrus fruit (*Citrus* spp.) as a pathway for the introduction of citrus canker disease (*Xanthomonas axonopodis* pv. *citri*[1])." That document concluded that "asymptomatic, commercially produced citrus fruit that has been treated with disinfectant dips and subject to other mitigations is not epidemiologically significant as a pathway for the introduction of citrus canker."

USDA-APHIS subsequently developed a risk management document USDA (2007b), "Movement of Commercially Packed Citrus Fruit from Citrus Canker Disease Quarantine Area Risk Management Analysis" based on the findings of the risk assessment. The risk management document evaluated the effectiveness of different management measures to address citrus canker disease risks associated with citrus fruit as a pathway. These analyses provided the basis for a rule, 7 CFR 301.75, with requirements for the interstate movement of fruit from areas where citrus canker occurred subject to certain requirements, including treatment of the fruit with disinfectants, inspection of the fruit to minimize the occurrence of diseased fruit in shipments, and restriction of shipments to non-citrus producing states.

New research summarized in two recent publications has provided additional evidence that addresses key uncertainties and which justifies revisiting our conclusions.

The first article, by Gottwald et al. (*In Press*), documents research on the survival of *Xanthomonas citri* subsp. *citri* (Xcc) on commercially produced and packed citrus fruit and the likelihood that such fruit could serve as a mechanism to spread the disease.

The second article, by Shiotani et al. (2009), documents research on the survival of Xcc on commercially produced mandarin fruits and the likelihood of spread of Xcc to trees from harvested mandarins.

Considering the above, USDA-APHIS has updated its 2007a analysis to include the following key relevant new findings: 1) post-harvest treatments are confirmed to substantially reduce the bacteria on fruit; 2) the viability of bacteria on fruit drops off significantly after it is harvested; 3) the low potential for spread from fruit to suitable hosts has now been reported by several sources (Civerolo, 1997; Gottwald et al., *In Press*, Shiotani et al., 2009); 4) discarded rinds of fruit do not spread the disease; and 5) fruit parts that are in direct contact with susceptible trees do not spread the disease.

This document, which supplements and updates the previous assessment, concludes that:

- asymptomatic fruit (treated or untreated) is not epidemiologically significant as a pathway for introducing citrus canker, and
- symptomatic fruit subjected to a packinghouse process that includes washing with disinfectants is also epidemiologically insignificant as a pathway for introducing citrus canker.

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[1] The classification of Xanthomonads was revised in 2006. The proper taxonomic designation for the pathogen that causes citrus canker is now *Xanthomonas citri* subsp. *citri* (Xcc); this pathogen was formerly known as *Xanthomonas axonopodis* pv. *citri* (Xac) (Euzeby, 2007; Schaad et al., 2006).

This supplemental analysis notes that minimizing the presence of lesions (i.e., minimizing symptomatic fruit) also reduces the risks of introducing Xcc via the fruit pathway and may be justified when typical packinghouse processes are unavailable or when the movement of symptomatic fruit to suitable areas (areas where the fruit has the potential to come into direct contact with suitable trees and high wind/rain conditions) within 24 hours of harvest are highly likely to occur.

Table of Contents

Introduction **1**
Phytosanitary Evidence Regarding Asymptomatic and Symptomatic Fruit as a Pathway ... **1**
(1) Infected/contaminated fruit are harvested 2
(2) Inoculum associated with fruit survives the packing/treatment process 2
(3) Inoculum associated with fruit survives shipment 3
(4) Fruit with inoculum go to an area with conditions suitable for infection 4
(5) Inoculum encounters a suitable host and conditions for disease development 4
Resolving Uncertainties **6**
Conclusion **7**
Authors **8**
References **8**

Introduction

This document is a supplement to USDA (2007a), "Evaluation of asymptomatic citrus fruit (*Citrus* spp.) as a pathway for the introduction of citrus canker disease (*Xanthomonas axonopodis* pv. *citri*)." USDA (2007a) concluded that "asymptomatic fruit is not epidemiologically significant as a pathway for introducing citrus canker when produced under the conditions of a systems approach." Since the completion of this document, new research has become available that further informs the risks associated with the movement of asymptomatic fruit, as well as the risks associated with the movement of symptomatic fruit as a viable pathway for the introduction and spread of citrus canker. The original risk assessment did not focus on the risks associated with the movement of symptomatic fruit, but the scientific literature analyzed in the previous document is applicable to characterizing the risks associated with the movement of symptomatic fruit.

The characterization of risk for any risk assessment is based on the scientific information available at the time of the assessment. As the body of scientific literature expands and changes, new research can often alter earlier conclusions regarding the risk level. The previous risk assessment noted, "Further research could strengthen the evidence surrounding the epidemiological significance (or lack thereof) of *Xanthomonas axonopodis pv. citri* (Xac) on symptomatic and asymptomatic commercial citrus fruit." In addition, it listed five key research needs that could better inform our conclusions regarding the risk level. A series of new research experiments summarized in two recent publications have addressed three key uncertainties identified in USDA (2007a). The new research justifies re-evaluating our previous conclusions in relation to several events identified as necessary for the pathogen to be introduced into a new area on commercial fruit, to ensure that they are consistent with the latest scientific information. This supplemental document will also focus for the first time on the epidemiological significance of symptomatic fruit as a viable pathway for the introduction of *Xanthomonas citri* subsp. *citri* (Xcc).

This document is designed to build directly upon the scientific information presented in the previous assessment (USDA, 2007a) and will primarily focus on new information. The reader is therefore also urged to consult USDA (2007a), "Evaluation of asymptomatic citrus fruit (*Citrus* spp.) as a pathway for the introduction of citrus canker disease (*Xanthomonas axonopodis* pv. *citri*)" as the primary reference for this supplement.

Phytosanitary Evidence Regarding Asymptomatic and Symptomatic Fruit as a Pathway

The previous risk assessment (USDA, 2007a) identified five key events required for citrus fruit to provide a pathway for the introduction of citrus canker disease. These events include:

(1) Infected/contaminated fruit are harvested;
(2) Inoculum associated with fruit survives the packing/treatment process;
(3) Inoculum associated with fruit survives shipment;
(4) Fruit with inoculum go to an area with conditions suitable for infection; and
(5) Inoculum encounters a suitable host and conditions for disease development.

These events are reviewed below with the aim of considering new information and the impacts of this information on our conclusions.

(1) Infected/contaminated fruit are harvested
Presently there is no new information that expands upon or alters our conclusions regarding the first event. We assume that Xcc is present in groves with active infections, or likely to be present in nearby groves from which the bacteria may be introduced. The research by Gottwald et al. (*In Press*) confirms that lesions on fruit are much less significant in disease spread than foliar lesions and that the viability of bacteria on fruit or associated with fruit lesions drops rapidly in the first 8 days after fruit is harvested and disappears completely 22 days after harvest whether or not the fruit is cleaned and treated.

(2) Inoculum associated with fruit survives the packing/treatment process
The assessment concluded that symptomatic fruit are unlikely to pass through the packinghouse process and that the standard packinghouse procedures and post-harvest treatments prescribed by the systems approach will remove and/or devitalize epiphytic populations of the pathogen to the extent that they become epidemiologically insignificant.

New research by Gottwald et al. (*In Press*) demonstrated that exposing apparently healthy fruit from infected citrus trees to a prewash with SOPP detergent followed by a wash with Cl reduced the survival of surface Xcc. Prewashing the fruit appears to be effective because it lowers the surface tension, which allows the Cl better access to bacteria and prevents the Cl from binding to debris or other surface contaminants. These results are not surprising given previous research by Brown and Schubert (1987) which showed that brushing increases the exposure of Xcc to detergents and other toxins.

In the research by Gottwald et al. (*In Press*) treating the fruit with only Cl did not greatly reduce surface bacteria populations. These results are in contrast to results obtained by Canteros et al. (2000), which showed that post-harvest treatments, such as chlorine or SOPP, guarantee the complete eradication of epiphytic Xcc on fruits without symptoms. The differing results could be attributed to differing bacteria loads on the fruit entering treatment. The amount of bacteria on the fruit can be affected by several factors, such as field control measures, the time of year that the fruit was harvested (differences in field bacteria load), and the amount of time the fruit were held before treatment.

Gottwald et al. (*In Press*) also examined the efficacy of the packinghouse process in reducing viable Xcc in lesions on symptomatic fruit. The packinghouse process was comprised of apparently healthy fruit from healthy trees, apparently healthy fruit from infected trees, and symptomatic fruit (run through the packing line separately and in combination with apparently healthy fruit). The fruit were either treated with Cl followed by detergent for 30-45 sec, washed with 2% SOPP for 45 sec using brushes, rinsed with water, and dipped in a shellac-based wax with fungicide; or prewashed with detergent, then treated with Cl, washed with 2% SOPP for 45 sec using brushes, rinsed with water, and dipped in a carnauba-based wax with fungicide. The experiments were designed to reflect current packinghouse processes in Florida, and differences in treatments reflect the changes in packinghouse protocols in the field. Both protocols significantly reduced (by approximately 50%) the ability of the lesions to produce viable bacteria.

The results of the new research lend additional support to the previous conclusion (USDA, 2007b) that standard packinghouse procedures and post-harvest treatments prescribed by the systems approach will remove and/or devitalize epiphytic populations of the pathogen, although Gottwald et al. (*In Press*) highlight the importance of a prewash. Based on Gottwald et al. (*In Press*), we also conclude that standard packinghouse procedures and post-harvest treatments prescribed by the systems approach will inactivate Xcc found in lesions on symptomatic fruit.

(3) Inoculum associated with fruit survives shipment

The assessment concluded that bacteria surviving the packinghouse process will have a high rate of mortality during shipping, and bacteria that survive on the fruit's surface or in lesions/injuries associated with fruit will not multiply or cause disease development after post-harvest treatment of the fruit.

New research by Shiotani et al. (2009) examined the length of time epiphytic Xcc survives on fruit surfaces under natural conditions. Apparently healthy fruit with no lesions were artificially contaminated by soaking the fruit in Xcc suspension. The fruit were placed in mesh bags and hung in the middle of naval orange trees in the field. After three days in the field no Xcc were recovered from the surface of the fruit. These results corroborate previous research that Xcc only survives for a limited amount of time [8-72 hours in sun and shade (Goto, 1969; Goto, 1962)]. Bacterial suspension of 10^6 cfu/ml sprayed on asymptomatic fruit sprayed, disappeared after five days at room temperature under lab conditions (Belasque and Rodrigues-Neto, 2000). The survival of epiphytic Xcc populations on asymptomatic fruit under natural conditions would be expected to be further reduced due to harsher environmental conditions.

New research by Gottwald et al. (*In Press*) demonstrated that the viability of bacteria on fruit and in lesions drops off significantly once fruit is harvested. Populations of Xcc in lesions of naturally infected fruit declined significantly over time, ceasing to produce viable Xcc after 22 days. Total bacteria populations (which included Xcc as well as other naturally occurring bacteria) declined in processed and unprocessed fruit. In all of the experimental scenarios, no harvested mature fruit ever developed new Xcc lesions. In addition, Gottwald et al. (*In Press*) inoculated wounds with Xcc bacteria. None of the inoculated wounds led to new Xcc lesions, and the bacteria levels in those wounds declined to undetectable levels within a few weeks after harvest.

Shiotani et al. (2009) wounded young susceptible Satsuma mandarin fruit still attached to the tree and inoculated them with Xcc. The fruit were allowed to mature on the tree and developed Xcc lesions. Upon harvesting, the Xcc level within the lesions were tested. A total of 24 lesions were tested and only three lesions contained detectable Xcc. The three lesions contained a low number of bacteria (lower then 3×10^3 cfu per lesion), which were short-lived in the lesions after harvest.

These results support previous research that the multiplication of Xcc bacteria associated with lesions is closely related to lesion expansion. In expanding lesions, Xcc bacteria multiply abundantly, but as lesion expansion ceases, bacteria multiplication noticeably decreases (Koizumi and Kuhara, 1982). Xcc bacteria do not increase in number on fruit once the fruit is

removed from the tree, but rather populations decline rapidly within the lesions of infected fruit following harvest (Civerolo, 1981; Koizumi, 1972).

The results of the new research support the previous conclusion in USDA (2007a) and also apply to symptomatic fruit:

- bacteria that survive the packing process will have a high rate of mortality during shipping; and,
- bacteria that survive on the fruit's surface or in lesions/injuries associated with fruit after post-harvest treatment will not multiply or cause disease development.

(4) Fruit with inoculum go to an area with conditions suitable for infection
Presently there is no new information that expands upon or leads us to change the conclusions of the fourth event. Although shipments of imported and domestically grown infected fruit to a suitable habitat are possible, only a relatively limited proportion of the citrus growing areas in the United States are at risk, as suitable conditions for the disease to develop occur mainly in Florida (e.g., Borchert et al., 2007).

(5) Inoculum encounters a suitable host and conditions for disease development
The assessment concluded that it is unlikely viable bacteria from an infected fruit would encounter a suitable host under the conditions required for disease development.

New research by Shiotani et al. (2009) examined the potential of fruit contaminated with Xcc to transmit that Xcc into the natural environment. Apparently healthy fruit with no lesions were artificially contaminated by soaking the fruit in a Xcc suspension. The fruit were placed in mesh bags and hung in the middle of naval orange trees in the field. After three days in the field, no Xcc were recovered from the surface of the fruit. In the three days that the fruit had detectable Xcc, no lesions developed on the leaves directly below the contaminated fruit.

Shiotani et al. (2009) also tested the potential of fruit with Xcc lesions to transmit that Xcc to into the natural environment. Young susceptible Satsuma mandarin fruit still attached to the tree were wounded and inoculated with a traceable strain of Xcc. The fruit were allowed to mature on the tree and developed Xcc lesions. Upon harvesting, symptomatic fruit were placed in polypropylene bangs and hung in the middle of naval orange trees. The Xcc in the harvested fruit did not transmit disease to the naval orange tree. Symptoms of Xcc developed on leaves below the experimental fruit in one tree, but the strain of Xcc used to inoculate the fruit was not found in the affected leaves, meaning that another strain of Xcc (likely occurring in the natural environment) infected the tree.

Gottwald et al. (*In Press*) examined several possible transmission scenarios to ascertain the potential of harvested fruit to transmit Xcc in the natural environment. In the first transmission scenario, symptomatic fruit that had undergone a typical packinghouse process were placed in cull piles with young susceptible trap trees surrounding them. The experiment was conducted in Florida and also in Argentina. In each experiment, treated symptomatic fruit were left in the field for several weeks under natural rain and wind conditions. None of the "trap" trees developed citrus canker.

Gottwald et al. (*In Press*) also tried to create a worst-case scenario by simulating spread with wind and rain events. In those experiments, severely infected fruit that were not put through the packinghouse process were either placed into elevated cull piles or suspended on vertical strings. Trap plants were placed around the infected fruit at distances of 0, 2, 5, and 10 m. An airboat fan was used to produce wind at various speeds (1, 10, and 25 m/sec), and a garden hose was used to simulate rain. In all the treatments, only one plant became infected with Xcc. The infected tree developed a single lesion at a wound site (likely created by the wind treatment). The infection occurred on a plant that was 0 m away from the infected fruit cull pile, and was subjected to 25 m/sec wind speed. The strength of this wind treatment is likely to only occur in strong storms, such as tropical storms. We note that a personal communication with the authors (December 2008) revealed that at the highest wind speed, the trap trees lost approximately 50% of their leaves.

During the Shiotani et al. (2009) experiment, a typhoon with winds as high as 50 m/sec struck the experimental Xcc experimental site. At the time of the storm, fruit artificially contaminated with a traceable strain of Xcc were hung in the field. Shiotani et al. (2009) were unable to recover the specific strain of Xcc from any of the fruit harvested after the storm.

In the another transmission scenario, Gottwald et al. (*In Press*) examined the potential for discarded infected citrus peel to infect susceptible citrus trees in nature. Fruit peels from severely infected fruit were placed in fiberglass mesh and placed either under a susceptible grapefruit tree in the shade or near the tree exposed to the sun. The peels were sampled weekly for viable Xcc. After one day in the field there were no longer any viable Xcc detected in the peel lesions, as bacteria isolated from the peels was unable to infect susceptible grapefruit trees via leaf injection-infiltration bioassays.

In the final transmission scenario, Gottwald et al. (*In Press*) examined the potential of Xcc bacteria to infect susceptible grapefruit plants when the fruit is forcefully broken and spread over the susceptible plants. This set of experiments used apparently healthy fruit from healthy trees, apparently healthy fruit from infected trees, and symptomatic fruit. Fruit were struck with a bat so that the flesh of the fruit would splatter over susceptible citrus plants. Three hundred total fruit (the juice and fruit fragments) were splattered over 300 plants and none developed Xcc symptoms or had Xcc bacteria recovered from the surface of the plants.

<u>We conclude that the results of the new research strongly indicate that both asymptomatic and symptomatic fruit produced commercially are not a likely pathway for the transmission of Xcc in the natural environment.</u> Xcc developed a single lesion on a leaf of a susceptible tree in a wound site, from Xcc bacteria transmitted from the fruit, in only one experiment under highly contrived conditions. In this case, the fruit were severely infected and subjected to extreme conditions of 25 m/sec wind with water. Even in this scenario, the fruit was only able to infect a tree immediately adjacent to the highly infested source and only when the untreated fruit pile was elevated from the ground.

These results support years of empirical data indicating that mature commercially produced citrus fruit are not a pathway for the transmission of the pathogen under most conditions likely to be encountered. The movement of commercial citrus fruit has not been associated with an

outbreak of the disease anywhere in the world.

Resolving Uncertainties

USDA (2007a) identified several key uncertainties that the scientific research was unable to clarify in 2007. Specifically it listed five key research needs that could better inform the risk assessment. As noted before, a series of new research experiments conducted by Gottwald et al. (*In Press*) and Shiotani et al. (2009) have addressed three of the key uncertainties identified in USDA (2007a).

Issue: Can symptomatic fruit that has been treated (with SOPP, chlorine, or other appropriate disinfectant) transmit the bacteria that cause the disease, (i.e., can disease be incited on healthy trees or seedlings from infected, symptomatic fruit that has been treated post-harvest)?

> *Importance:* Phytosanitary requirements are not justified if treated fruit with lesions are not a pathway.

Gottwald et al. (*In Press*) demonstrated that symptomatic fruit that has undergone a typical packinghouse process (the fruit were either treated with Cl followed by detergent for 30-45 sec, washed, using brushes, with 2% SOPP for 45 sec, rinsed with water, and dipped in a shellac-based wax with fungicide; or prewashed with detergent, then treated with Cl, washed, using brushes, with 2% SOPP for 45 sec, rinsed with water, and dipped in a carnauba-based wax with fungicide), did not transmit Xcc. This research strongly indicates that treated symptomatic fruit are not a pathway for the transmission of Xcc in the natural environment.

Issue: How effective are different products at reducing the biological activity of bacteria in lesions, (i.e., what is the efficacy of various post-harvest treatments (e.g., SOPP, chlorine, etc) at rendering symptomatic fruit epidemiologically insignificant)?

> *Importance:* Chlorine is a common standard; it is also highly unstable. Alternative products and their activity need to be better understood.

Gottwald et al. (*In Press*) demonstrated that exposing apparently healthy fruit from infected citrus trees to a prewash with SOPP detergent followed by a wash with Cl reduced the survival of surface Xcc. Symptomatic fruit either treated with Cl followed by detergent for 30-45 sec, washed with 2% SOPP for 45 sec, rinsed with water, and dipped in a shellac-based wax with fungicide; or prewashed with detergent, then treated with Cl, washed with 2% SOPP for 45 sec using brushes, rinsed with water, and dipped in a carnauba-based wax with fungicide, significantly reduced (by approximately 50%) the ability of the lesions to produce bacteria.

Issue: How long after post-harvest treatment can Xcc be recovered from asymptomatic fruit.

> *Importance:* The propagules are not very long-lived outside of suitable conditions. As fruit is treated and dried and then stored, there may be sufficient

mortality (even in fruit with lesions) to render such fruit epidemiologically insignificant (not a pathway) because of the time this fruit is exposed to unsuitable conditions.

New research by Shiotani et al. (2009) demonstrated that after three days under natural conditions no Xcc bacteria were recovered from the surface of the artificially contaminated fruit. These results corroborate previous research that Xcc bacteria only survives for a limited amount of time [8-72 hours in sun and shade (Goto, 1969; Goto, 1962)]. Bacterial suspension of 10^6 cfu/ml sprayed on asymptomatic fruit sprayed, disappeared after five days at room temperature under lab conditions (Belasque and Rodrigues-Neto, 2000). The survival of epiphytic Xcc populations on asymptomatic fruit under natural conditions would be expected to be further reduced due to harsher environmental conditions.

In addition, new research by Gottwald et al. (*In Press*) demonstrated that the viability of bacteria on fruit and in lesions drops off significantly once fruit is harvested. Populations of Xcc declined rapidly over time, ceasing to produce viable Xcc after 22 days. Total bacteria populations (which included Xcc as well as other naturally occurring bacteria) declined in both processed and unprocessed fruit.

Conclusions

The above information in conjunction with the evidence presented in USDA (2007a), "Evaluation of asymptomatic citrus fruit (*Citrus* spp.) as a pathway for the introduction of citrus canker disease (*Xanthomonas axonopodis* pv. *citri*)," consistently argue that asymptomatic fruit (treated or untreated) is not epidemiologically significant as a pathway for introducing citrus canker and symptomatic fruit presents a low risk of introducing citrus canker. While occurring under extreme, contrived conditions there was one instance of untreated highly symptomatic citrus fruit transmitting Xcc bacteria to a susceptible plant. This occurred under highly contrived conditions: forced rain; severely infected fruit, infected fruit elevated above the ground; infected fruit immediately adjacent to highly susceptible trees; very high wind conditions; and hot, humid subtropical Florida Summer environment. APHIS concludes, based on all of the available literature, that if the fruit used in the forced wind and rain experiment were treated in the packinghouse (with a prewash, Cl dip, washing with brushes, water dip, and then dipped in wax with fungicide) it is highly unlikely that there would have been enough remaining Xcc to successfully transfer from the fruit and infect a susceptible host.

Therefore if symptomatic fruit are subjected to a packinghouse process then the symptomatic fruit also become epidemiologically insignificant as a pathway for introducing citrus canker even under extreme conditions.

Minimizing the presence of lesions (i.e., minimizing symptomatic fruit) also reduces the risks of introducing Xcc via the fruit pathway. A requirement for minimal presence of lesions on fruit may be justifiable only when typical packinghouse process are unavailable or when the movement of symptomatic fruit to suitable areas (areas where the fruit has the potential to come into direct contact with suitable hosts and high wind/rain conditions) within 24 hours of harvest are highly likely to occur.

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